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In vitro induction of genetic variability in cotton (*Gossypium* spp.)

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Summary. Through the in vitro culture of excised embryos and ovules, interspecific hybrids have been obtained from cultivated and wild species of *Gossypium.* The hybrids matured upon transfer to the field. The anthers, ovules and embryos from both the diploid (2n=26) and tetraploid (2n=52) species underwent proliferation, and this response was genotypic. The diploid species invariably showed profuse callusing in comparison with the tetraploid. The callus showed various chromosome numbers, ranging from haploids to hexaploids, and from high polyploidy to aneuploidy. Hybrid callus culture may augment the genetic variability by providing a means for obtaining genetic exchange in interspecific hybrids. The implications of the in vitro induction of genetic variability for cotton improvement are discussed.

Key words: Cotton – Genetic variability – Tissue culture – Wide hybridization

Introduction

Cotton (*Gossypium* spp.), the most important source of textile fibre, has 35 wild and 4 cultivated species, both diploid (2n=26) and tetraploid (2n=52). Though such desirable traits as quality of fibre and resistance to various diseases and pests are present in the wild species, by using conventional methods the incorporation of these characters into cultivated species has not had much success. This is mainly due to the incompatible barriers, such as embryo and endosperm breakdown (Weaver 1958; Pundir 1972). Thus, various in vitro methods have been employed to generate genetic diversity (Bajaj and Gill 1985). The present report summarizes the results on the successful recovery of interspecific hybrids amongst various cultivated and wild species through the culture of hybrid embryos and ovules. In addition, cytological data on the callus raised from the cultured anthers, ovules and embryos, showing a wide range of variability are presented.

Materials and methods

Three cultivated species (*Gossypium arboreum, G. herbaceum, G. hirsutum*) and two wild species (*G. anomalum, G. stocksii*) of cotton grown in the Experimental Fields of the Punjab Agricultural University, Ludhiana, were employed for the present investigation.

1 Interspecific hybridization

The flower buds were emasculated in the evenings, and the pollinations were done the next morning. The pollinated buds were treated with a solution of naphthalene acetic acid (100 mg/l) and gibberellic acid (50 mg/l) to prevent early abscission, and thus to encourage their further development. The developing bolls were harvested 3 and 15 days after pollination (DAP) for ovule and embryo culture, respectively.

For ovule culture the bolls from various crosses (Table 1) were excised, dipped in ethanol and flame-sterilized. The ovules were dissected out and cultured on liquid (Stewart and Hsu 1978), as well as agar-solidified MS medium (Murashige and Skoog 1962) supplemented with IAA (1.5 mg/l) + kinetin (0.5 mg/l) + casein hydrolysate (500 mg/l). About 25 ovules were transferred to 100 ml Erlenmeyer flasks containing 20 ml of the liquid medium, and 6–8 ovules were cultured in 150×25 mm test tubes containing agar-solidified medium. Likewise, the hybrid embryos were excised from the 15 DAP ovules and cultured (Gill and Bajaj 1984). The cultures were maintained at 27–30 °C. The hybrid plants were transferred to pots in the greenhouse, and to the field.

2 Cytological studies of callus

The callus was initiated from excised anthers, ovules and embryos. Anthers of *G. arboreum* at various stages of develop-

Table 1. Growth response of the excised ovules of various interspecific crosses of *Gossypium* cultured 3 days after pollination on MS+IAA (1.5 mg/l)+kinetin (0.5 mg/l)+CH (250 mg/l)

Hybrid	No. of ovules cultured	No. of ovules germinated	% age germination
1. *G. arboreum* × *G. stocksii*	145	41	28
2. *G. herbaceum* × *G. stocksii*	72	16	22.2
3. *G. arboreum* × *G. anomalum*	80	17	19.1
4. *G. arboreum* × *G. hirsutum*	68	23	34.7

ment were cultured on MS+NAA (2 mg/l)+BAP (1 mg/l), whereas ovules and young embryos proliferated well on Smith et al. (1977) medium (MS+IAA 2 mg/l+kin 0.5 mg/l)+glucose (3%). The callus was periodically subcultured, and maintained on MS+NAA (2 mg/l)+BAP (1 mg/l). The fast-growing callus (7–10 days after subculturing) was fixed in 1:3 acetic acid ethanol. In some cases callus was pretreated with a saturated solution of p-dichlorobenzene for 3 h at 16 °C and then fixed. After fixation, the callus was transferred to a 2% solution of acetic orcein stain, and kept at room temperature for 10 days. Slides were prepared by squashing a small piece of callus in 45% acetic acid. The preparations were sealed with parafin wax. At least 500 dividing cells were observed for chromosome counts in each experiment.

Results

1 Culture of hybrid ovules

The hybrid ovules of various crosses (Table 1) cultured 3 DAP on MS+IAA (1.5 mg/l)+kin (0.5 mg/l)+CH (250 mg/l) attained double their size in 4–6 days of culture, and the callus formation started within one week. There was profuse proliferation in some cases, and the callus was soft and friable (Fig. 1 A–D). The callus formed from hybrid ovules with *G. arboreum* cv. G.27 as the female parent was pinkish, whereas the ovules with *G. herbaceum* as the female parent formed creamy-white callus. The ovules continued to grow along with the mass of callus, and were either embedded in the callus, or were lying on its surface. The ovules started to germinate after about 50 days of culture (Fig. 1 D). At the time of germination, the size of the ovules was smaller than the ones developed in situ. In most cases the cotyledons were either malformed or did not develop. A maximum number of seedlings were produced in *G. arboreum* × *G. hirsutum*, however, their survival was low (Fig. 1 E). The seedlings obtained from *G. arboreum* × *G. anomalum* ovules were vigorous, and showed the highest survival rate. These seedlings, upon transfer to a fresh medium containing half strength MS+1% sucrose, continued further growth. The seedlings when transplanted into pots and field matured. The root tip of *G. arboreum* × *G. hirsutum* seedlings showed a triploid (3x=3n=39) chromosome number (Fig. 2 C). All the hybrids were intermediate between the two parents.

2 Culture of hybrid embryos

The hybrid embryos excised from 15 DAP ovules, and cultured on MS+IAA (1.5 mg/l)+kin (0.5 mg/l)+CH (250 mg/l) when kept in the dark started to elongate after two weeks. Upon transfer to light the cotyledons turned green, the first leaf appeared within 30–35 days and the embryos developed into small seedlings (Fig. 1 F). Their growth responses, and % survival upon transfer to soil, however, varied in different crosses (Gill and Bajaj 1984). The *G. arboreum* × *G. anomalum* hybrids showed very vigorous growth (Fig. 1 H, Fig. 3) and produced flowers throughout the year. All the hybrids involving wild species were perennial in habit.

3 Chromosomal variations in callus culture

The callus cultures raised from the embryos, ovules, anthers and hypocotyl segments of *G. arboreum* and *G. herbaceum* were fast growing. In both species the ovule-derived callus grew faster than that from the anthers and the hypocotyl. *G. herbaceum* callus was whitish, whereas that of *G. arboreum* was reddish – the colour of the donor variety G 27. The extent of genetic variability in terms of chromosome numbers is shown in Figs. 2 and 4. The basic chromosome number in both species under study is 26 (2n), however, in the callus, the number ranged for haploid (13) to a highly polyploid (> 104).

Hypocotyl-derived callus. The ploidy level of callus cells varied from haploid to more than hexaploid (6n=78). The frequency of haploid cells was quite low (0.74%). Most of the cells had a diploid (45.2%) or tetraploid (40.5%) number. Triploid (4.6%), pentaploid (2.6%), hexaploid (4.4%) or even a higher ploidy than hexaploid (2.9%) cells were also observed (Fig. 4 A).

Ovule-derived callus. The range of genetic variability was greater in the ovule-derived callus of *G. herbaceum* than in those of *G. arboreum*. In *G. herbaceum* callus, the haploid, triploid, pentaploid, hexaploid and higher than hexaploid were present in higher frequency, whereas *G. arboreum* callus had a higher number of diploid and tetraploid cells. In general, ovule-derived

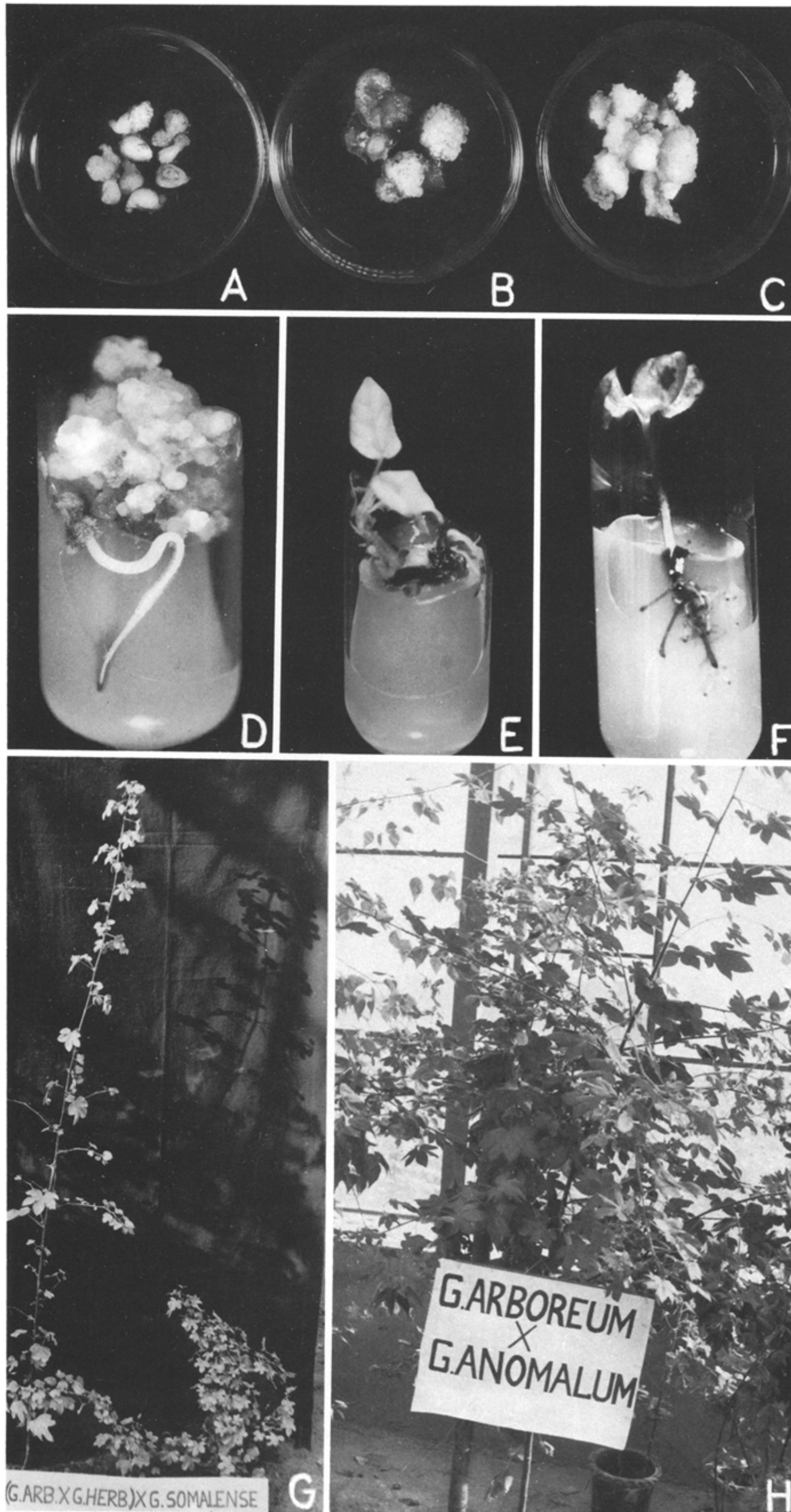

Fig. 1 A–H. Regeneration of plants from in vitro cultured ovules and embryos of various interspecific crosses in *Gossypium*. **A–C** Growth of ovules of *G. hirsutum* × *G. arboreum* (cultured 3 days after pollination) on Stewart and Hsu's (1978) medium containing IAA (0.5 mg/l) after 14, 21 and 28 days of culture, respectively; **D** *G. arboreum* × *G. hirsutum* ovules (3 DAP) after 50 days of culture on MS + IAA (1.5 mg/l) + kin (0.5 mg/l) + CH (250 mg/l); note the profuse proliferation, and the radicular •root; **E** A plantlet obtained from a *G. arboreum* × *G. hirsutum* ovule (3 DAP) after 85 days of culture on MS + IAA (1 mg/l) + kin (0.2 mg/l) + CH (250 mg/l); **F** A plantlet formed from *G. arboreum* × *G. stocksii* embryos (15 DAP) after 35 days of culture on MS + IAA (1.5 mg/l) + kin (0.5 mg/l) + CH(250 mg/l)showing root and shoot formation; **G** Diversity in plants derived from the embryos of a three way cross (*G. arboreum* × *G. herbaceum)* × *G. somalense* (8-months old); **H** *G. arboreum* × *G. anomalum* hybrid at flowering stage after eight months of culture (at 3 months it was transfered to the greenhouse)

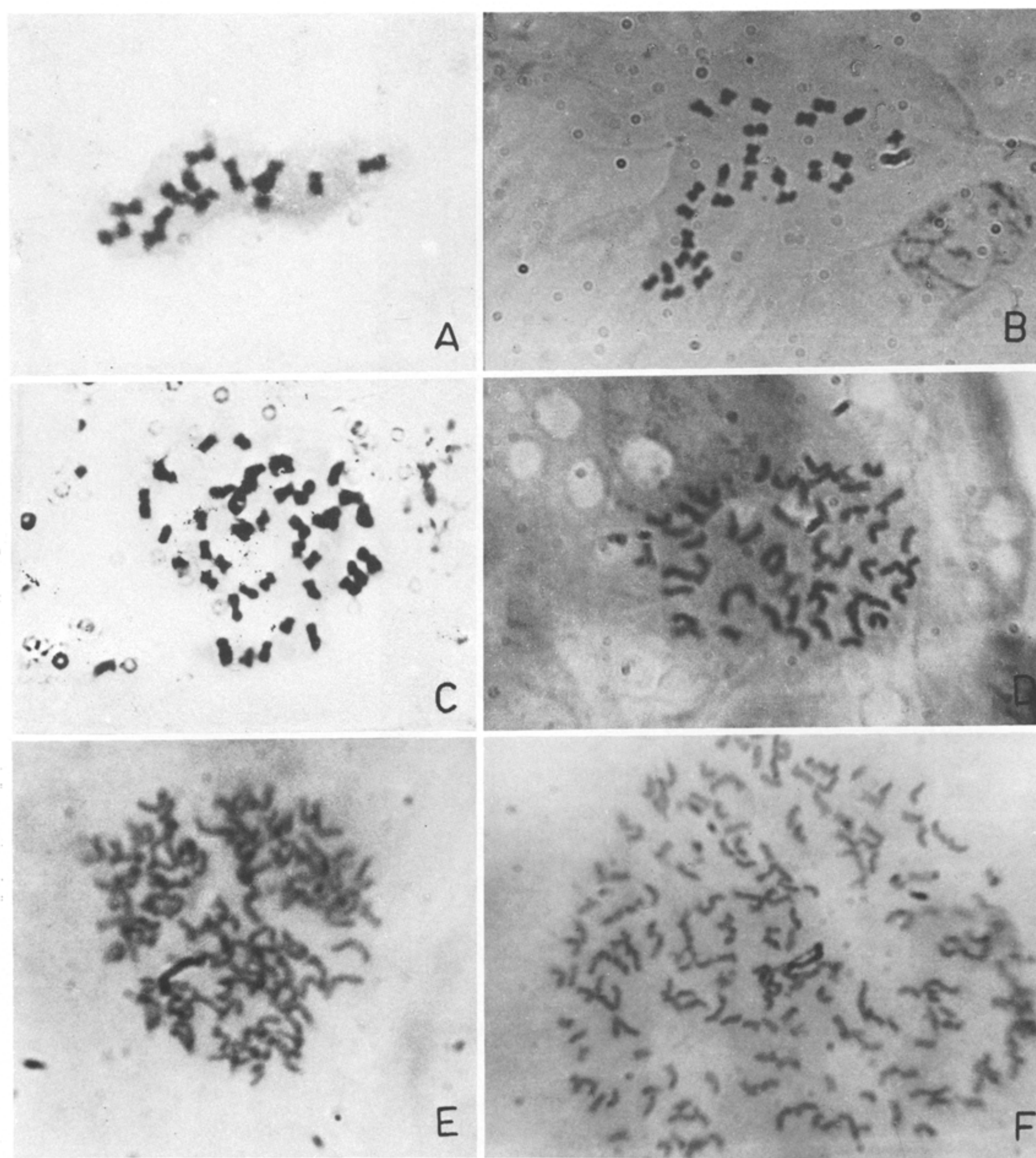

Fig. 2A–F. Genetic variability in ovule and embryo-derived callus, showing chromosome numbers varying from haploid to highly polyploid. **A** haploid ($n=13$); **B** diploid ($2n=26$); **C** triploid ($3n=39$); **D** tetraploid ($4n=52$); **E** polyploid ($6n=78$); **F** highly polyploid

callus of both species contained cells with more variable number of chromosomes than the hypocotyl –, or anther-derived callus (Fig. 4B).

Anther-derived callus. The callus cells obtained from young anthers (Bajaj 1982) showed a wide range of chromosome numbers, varying from haploid (n = 13) to hexaploid (6x = 78). In most of the cells (73.1%), the number was close to the diploid (38.3%) or tetraploid (34.8%) level. Cells with a haploid (3.5%), triploid (15.1%), pentaploid (6.8%) and hexaploid (1.5%) number of chromosomes were present in relatively low frequencies (Fig. 4C) whereas cells with more than 78 chromosomes were not present.

Hybrid embryo-derived callus. The embryos of a cross, *G. hirsutum* × *G. arboreum,* cultured on Smith et al. medium (1977) proliferated to form callus. This was then subcultured periodically on MS + NAA (2 mg/l) + BAP (0.5 mg/l). The callus was creamy-white, soft and friable. Cytological examination of one-year-old callus showed a wide range of chromosome number, varying

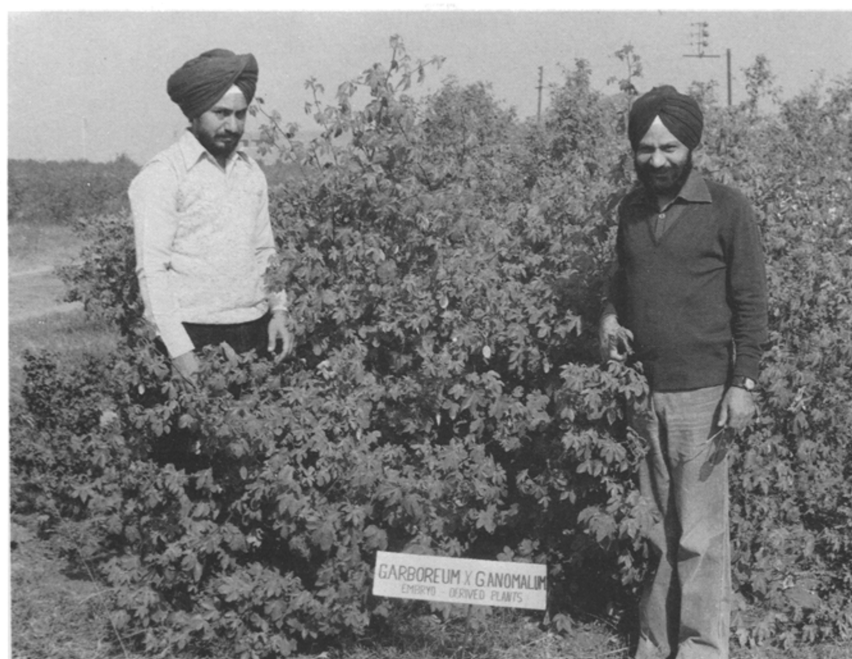

Fig. 3. Embryo-derived hybrid plants (*G. arboreum* × *G. anomalum*) 6 months after transfer to the field (total time taken from embryo culture to flowering stage is 11 months)

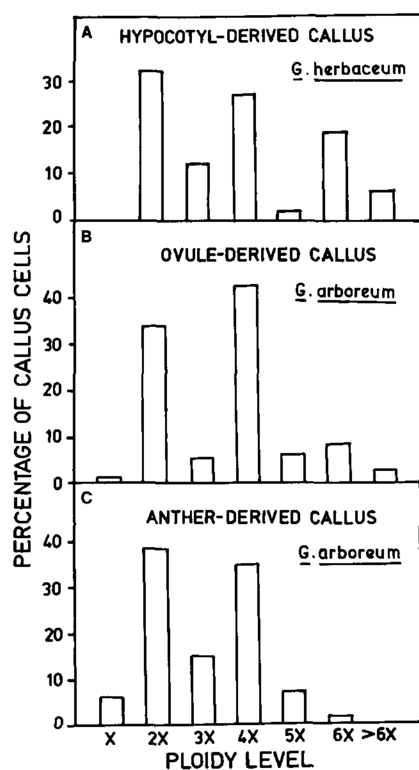

Fig. 4. Histograms showing the extent of genetic variability of callus cells obtained from excised hypocotyl, ovules and anthers of various species of *Gossypium* (data based on 500 dividing cells in each case)

Table 2. Variability in chromosome number in hybrid callus cells obtained from embryos of a cross *Gossypium hirsutum* ($2n=52$) × *G. arboreum* ($2n=26$). Data based on 477 dividing cells

Ser. no.	Chromosome no.	No. of cells studied	% age
1.	25	7	1.47
2.	26–29	70	14.67
3.	30–33	105	22.01
4.	34–37	161	33.75
5.	38–41	123	25.79
6.	42–45	7	1.47
7.	45	4	0.84

from less than 26 to more than 45 (Table 2). However, the majority of the cells belonged to the category 26–41. The cells with less than 26, and more than 45 chromosomes were rare, i.e. 1.4% and 0.84%, respectively. No polyploids were observed.

Discussion

The success of any crop improvement program depends on the extent of genetic variability in the base population. In this regard tissue cultures are known to be a rich source (D'Amato 1977; Skirvin 1978). The callus tissues on prolonged culturing undergo endomitosis, chromosome loss, polyploidy, aneuploidy, mutations and other genetic changes (Larkin and

Scowcroft 1981). Though most of such changes may not be of much significance, yet there may be some which can be selected and isolated. This phenomenon has especially been exploited in sugarcane (Liu and Chen 1976) and potato (Shepard et al. 1980) for the improvement of these crops. In the present investigation on cotton, two in vitro approaches, i.e. interspecific hybridization, and callus cultures have provided a wide range of material ranging from haploids to highly polyploid and aneuploids.

Wild species belonging to the genus *Gossypium,* possess a number of useful traits such as insect-pest resistance, disease resistance, drought resistance, and superior fibre qualities. However, due to various incompatibility barriers, it has not been possible to introduce particular genes from wild species into commercial crop cultivars. Even if hybridization between different species is effective, due to small structural differences in the chromosomes of different species (Stephens 1950), pairing and subsequent exchange of genetic material between chromosomes of two species is prevented. In subsequent generations of selfing distorted segregation ratios are obtained (Gerstel and Phillips 1958), and recombinants are produced in extremely low frequency. If a small segment of alien genome carrying desirable genes is transferred to the chromosomes of the cultivated species, then that trait can be stabilized in commercial crop cultivars. It has been speculated (Larkin and Scowcroft 1981) that tissue culture may generate environments for enhancing chromosome breakage and reunion events, and thus a tissue culture cycle of the hybrid material may provide the means for obtaining the genetic exchange needed between two genomes in the interspecific hybrid. The hybrid callus may enhance the frequency of requisite exchange (Larkin and Scowcroft 1981).

It is interesting to point out that in the present study callus obtained from the triploid hybrid embryo ($3n=39$) of a cross *G. arboreum* × *G. hirsutum* showed a much wider diversity in chromosome number varying from 25–45. The occurrence of cells with less chromosomes may be due to laggards at anaphase. The very low frequency of cells with more than 39 chromosomes can also be accounted for by laggards, rather than by endomitotis or other types of polyploidization.

To conclude, it may be emphasized that the in vitro production of interspecific hybrids in *Gossypium* through embryo and ovule culture has enabled the synthesis of wild and cultivated cotton. The genetic pool will be augmented by the callus, which would further enhance the genetic variability in the base population. The hybrid callus may provide the means for obtaining the genetic exchange needed between two genomes in the interspecific hybrids. Hybrid callus which is a rich source of genetic diversity needs to be exploited for crop improvement.

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